

1991

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Knorr, David A. and Morris, Thomas Jack, "Origin and Evolution of Defective Interfering RNAs of Tomato Bushy Stunt Virus" (1991). *Faculty Publications in the Biological Sciences*. 415.
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ORIGIN AND EVOLUTION OF DEFECTIVE INTERFERING RNAs OF TOMATO BUSHY STUNT VIRUS

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INTRODUCTION

Viruses with defective genomes have been identified in association with virtually every major family of viruses and have been widely utilized as tools for investigating virus functions in animal cell culture systems (Perrault, 1981). It is generally thought that defective interfering viruses (DIs) arise through deletion, rearrangement, or recombination of a competent viral genome. DIs lack the ability for independent existence relying on their parental helper viruses to supply factors required for replication, maturation, and/or encapsidation (Huang and Baltimore, 1977). The interference attributed to DIs is thought to result from competition with the helper virus for factors required in *trans* for replication and/or encapsidation (Schlesinger, 1988). It is these features that have made DIs very useful tools for mapping viral signals required for replication and packaging (e.g. Levis, *et al.*, 1986). The DIs associated with animal viruses have usually been detected after serial passage of virus at high multiplicities of infection (m.o.i.) in cell cultures. It has also been noted that maintenance in continuous passage results in fluctuations of helper and DI which reflect the relative abilities of each to interfere or support the other (Huang, 1988). DIs have also been detected in natural virus infections and, although there is some debate, may function *in vivo* to modulate virus diseases and allow more persistent infections (Huang, 1988).

The majority of plant viruses have positive-sense, single-stranded RNA genomes. Additional RNA components capable of modulating symptoms have most commonly been associated with either satellite viruses or satellite RNAs. Satellite viruses differ from satellite RNAs by encoding their own capsid proteins, whereas satellite RNAs are encapsidated by their specific helper virus. Satellite RNAs are relatively common among plant viruses, having been found in association with at least 24 members in six virus groups (Francki, 1985). Unlike DIs, satellite RNAs in general share little sequence similarity with their specific helper virus. The one exception is the chimeric satellite RNA-C of turnip crinkle virus (Simon, 1988). Satellite viruses and satellite RNAs have been shown to both intensify as well as attenuate symptoms normally expressed by their specific virus helpers (Kaper, and Collmer, 1988, Simon, 1988).

In contrast to the many reports of satellites, few authentic DIs have been identified among plant viruses. These accounts include the presence of DI-like particles associated with the negative-stranded plant rhabdoviruses (Adam *et al.*, 1983; Ishmail and Milner, 1988) and the bunyavirus-like tomato spotted wilt virus (Verkleij and Peters, 1983). DI-like RNA components have also been reported for wound tumor

virus, a plant reovirus (Nuss, 1988). Among the positive, single-stranded RNA plant viruses, authentic DIs have been identified for only two viruses: tomato bushy stunt virus (TBSV) (Hillman *et al.*, 1987; Morris *et al.*, 1989); and turnip crinkle virus (TCV) (Li *et al.*, 1989).

TBSV and TCV are members of two closely related groups of plant viruses, the tombusviruses and the carmoviruses respectively (see Martelli *et al.*, 1988 and Morris *et al.*, 1988 for reviews). Both viruses are good model systems for studying small RNA virus and DI RNA molecular biology because each has been well-defined biologically and detailed structural studies have been performed on both viruses. In addition, the genome of each virus has been cloned, entirely sequenced, and engineered to produce infectious RNA transcripts *in vitro* (Carrington *et al.*, 1989; Heaton, *et al.*, 1989; Hearne, *et al.*, 1990). The reader is referred to these papers for details on the genomic organizations of these two viruses. The features of the TBSV genome organization important in understanding the origins of associated DI RNAs are summarized in Figure 5.

In this paper we will focus on the derivation, molecular characterization, and evolution of TBSV-associated DIs. We will also discuss the possibilities for using plant virus DIs as a general method for controlling virus diseases.

DISCOVERY OF DI RNAs IN TOMBUSVIRUSES

In studies with the cherry strain of TBSV, it was observed that plants inoculated with concentrated virus preparations tended to develop a less-severe, more persistent disease syndrome than plants inoculated with diluted virus. This interference was found to be associated with a novel small RNA species (400b) present in the virus isolate (Hillman, *et al.*, 1985). This observation was similar to the "autointerference" phenomenon in some animal virus systems attributed to the presence of DIs (Huang, 1988) and to the effects of some satellite RNAs associated with RNA plant viruses. Although a satellite RNA had been identified in association with another tombusvirus at that time (Gallitelli and Hull, 1985), it was not suspected to be involved in the TBSV-cherry system because northern hybridizations consistently indicated that the low molecular weight (LMW) RNA in our culture hybridized with the viral genome. Subsequent sequencing of clones of TBSV-associated small RNAs in conjunction with genomic sequencing clearly demonstrated that the interference-associated RNAs were collinear deletion mutants of the helper virus comprised of sequences derived from 5' proximal, internal, and 3' proximal regions of the genome (Hillman, *et al.*, 1987). Plant infection studies demonstrated that the DI RNA was responsible for the symptom attenuation as well as decreased viral replication and reduced virus accumulation in whole plants (Hillman, 1986). More recent studies using plant cell protoplasts have shown that TBSV DIs specifically interfere with the replication of helper virus as judged by reduced incorporation of ³H-uridine into viral and viral-specific, sub-genomic RNA species (Jones, *et al.*, 1990). These features are consistent with the definition of defective interfering viruses and establish the first definitive proof for the existence of DIs in association with a plant virus.

DE NOVO GENERATION OF TBSV DI RNAs

A previous report had suggested that the satellite RNA associated with CyRSV was generated upon serial passage of the virus in plants (Gallitelli and Hull, 1985). In our laboratory, however, LMW RNA species suggestive of DIs were readily detected after serial passage of several different tombusviruses including artichoke mottle crinkle virus, pelargonium leaf curl virus, petunia asteroid mosaic virus, and a field isolate of the BS3 strain of TBSV (Morris, unpublished). Curiosity about the possible *de novo* generation of these DIs prompted a more definitive set of passage experiments. Sixteen lines of a DI-free isolate of TBSV-cherry were generated by inoculating single local lesions from *Nicotiana glutinosa* onto each of 8 *N. clevelandii* and 8 *N. benthamiana* plants. Each of the independent lines of TBSV were passed at 7 day intervals, 6 lines for each host at high m.o.i. (undiluted sap) and 2 lines at

low m.o.i. (buffer-diluted sap). This approach was similar to that routinely employed to generate DIs in animal cell lines. After each passage, infected plants were observed for alterations in symptom development, and inoculated leaves were analyzed for ssRNA, dsRNA, and virus production. An advantage in using an intact host rather than cell cultures was the ability to detect the presence of DIs by observing attenuation of disease symptoms. In the course of the passage experiment, symptom attenuation was evident in some of the high m.o.i. passed lines as early as passage 3 and in all such lines by passage 10 but not in any of the 4 lines passed at low m.o.i. The symptom attenuation was also correlated with LMW RNAs of approximately 400-600b present in viral RNA preparations analyzed by polyacrylamide gel electrophoresis (PAGE) of each of the independent virus lines passed at high m.o.i. and in none of lines passed at low m.o.i. (Figure 1). These results strongly support the contention that DIs arise spontaneously upon host passage because distinctively sized DI species appeared in each of the high m.o.i. lines at different stages of the passage experiment. More definitive proof for *de novo* generation of TBSV DI RNAs has since come from the identification of DI species in lines derived from clones of TBSV by inoculation of infectious transcripts of the viral genome (Knorr, in preparation.).

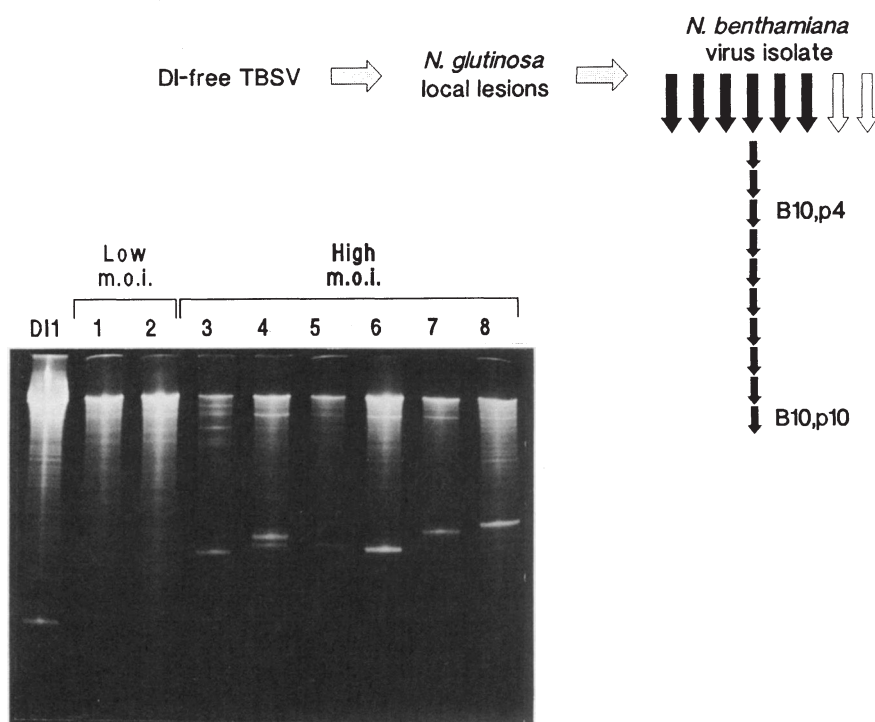


Figure 1. Strategy for *de novo* generation of TBSV DI RNAs. The diagram illustrates how independent TBSV isolates were obtained in *N. benthamiana*. Black vertical arrows indicate high m.o.i. passage lines; white arrows indicate two lines passed at low m.o.i. Each independent line was passed ten times (indicated by the smaller black arrows for the B10 line). Results from the B10 passage are presented in Figure 4. The lower panel shows an ethidium bromide-stained gel of virion RNAs after the tenth passage from the TBSV isolates passed in *N. clevelandii*. DI1 is a 400b spontaneous DI originally described (Hillman, *et al.* (1987)). Lanes 1 and 2 are from the low m.o.i. lines, 3-8 are from high m.o.i. lines.

MOLECULAR CHARACTERIZATION OF A DI POPULATION

The TBSV DI RNAs generated in each of the high m.o.i. lines of the passage experiment were initially identified by northern analysis using a TBSV specific probe. Typical results for one of the lines generated by passage in *N. benthamiana* (designated DI B10) are presented in Figure 2. Consistent with the properties of the originally characterized TBSV DIs, the B10 DI species could be readily detected in the 2.5 M lithium chloride soluble fraction of infected tissue extracts as well as in the virion RNA preparations.

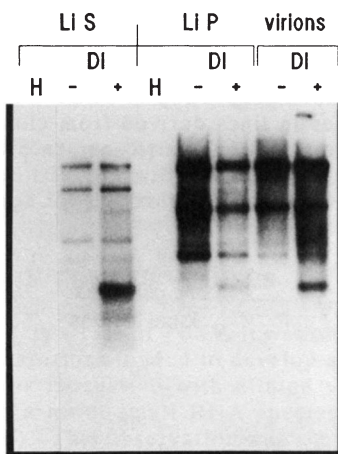


Figure 2. Northern analysis of DI RNAs. Total nucleic acids were extracted from healthy (H), TBSV (-), and TBSV-DI (+) infected plants, treated with 2.5 M LiCl and DNase, then separated into soluble (LiS) and pellet (LiP) fractions. The LiCl products and virions RNAs a TBSV isolate with (+) and without (-) DI RNAs were then separated by non-denaturing PAGE. The RNAs were transferred to a nylon membrane and probed with [32 P]-labelled TBSV-cDNA.

A series of cDNA clones of the B10 line then was produced in phagemid vectors by standard protocols using RNA templates purified from virions (Knorr, in prep). The complete nucleotide sequences for six apparently full-length cDNA clones of the B10 DI isolate were determined using the di-deoxynucleotide chain terminating method. The sequence analysis confirmed the DI nature of each of the clones which showed marked similarity. Each consisted of the same four regions of sequence derived entirely from the helper virus genome. The four regions of sequence maintained in each DI were: (I) the complete 168 nucleotide TBSV 5' leader sequence, including the initiator methionine codon; (II) a region of between 173 to 234 nucleotides (nt 1285 to 1481-1523) from the putative polymerase gene; (III) approximately 70 nt containing the 3' terminus of the viral P19 and P22 open reading frames (ORFs); and (IV) the extreme 3' terminal 130 nt of non-translated viral sequence (see the diagram in Fig. 5). Minor variations in each sequence consisting of single base changes and small deletions were detected. However, conservation of the deletion junctions were maintained between each region of retained sequence suggesting that this population evolved either from a common progenitor, or by some inflexible molecular mechanism. It is interesting that at each sequence junction there is an ambiguity of one to four nucleotides. At the junction between regions II and III, the sequence 'AGAA' could be derived from genomic sequences at either side of the deletion. The same final sequence motif (AGAA) was present between regions I and II, and between III and IV, in another DI population analyzed later. It is also worth mentioning here that the same general regions of conserved sequence were identified

in the first DI to be sequenced (Hillman *et al.*, 1987) and in another of the *de novo* generated lines derived by passage in *N. cleveandii* that was also cloned and sequenced (see the C6-line identified in Fig. 5).

It is possible that DI RNAs translate proteins that are in some way involved in attenuating symptoms or virus replication. To examine this possibility, each sequence was analyzed for the presence of coding regions. The largest ORFs encoded by the B10 DIs are 31 and 34 amino acids respectively. They share no apparent similarity with virus, or other proteins, nor are they strictly conserved in each DI. Experiments with an additional set of DI clones indicates even more variation in these two ORFs. Also, previous attempts to translate DIs *in vitro* were negative (Brad Hillman, unpublished). Therefore, it seems unlikely that the mechanism by which DIs attenuate symptoms involves activity of a DI-encoded protein. It is possible, however, that sequestering of ribosomes by DI RNAs, or a similar activity, may affect disease outcome.

INFECTIVITY OF CLONED TBSV DIs

Biological activity of DI sequences was assessed by making RNA transcripts *in vitro* from the cloned cDNAs and co-inoculating them with DI-free TBSV (Figure 3). In order to accomplish this, however, the inserts had to be modified. The 5' termini

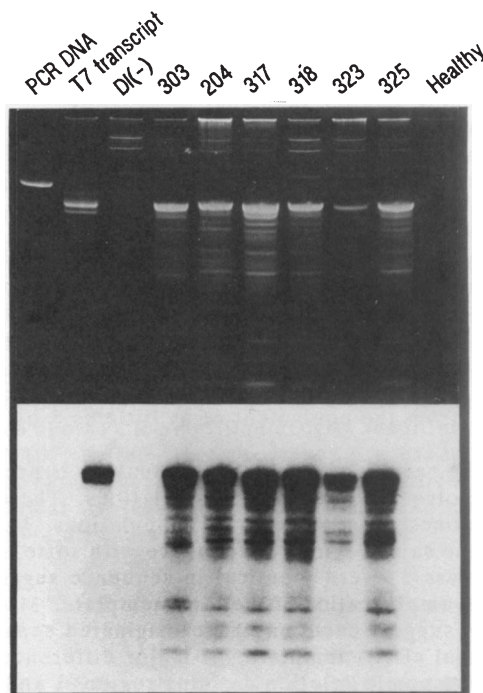


Figure 3. Infectivity of transcripts from TBSV DI B10 clones. PCR-amplified cDNAs were transcribed *in vitro* and inoculated onto *N. cleveandii* together with TBSV genomic RNA. Plants inoculated with DI transcripts survived, whereas those inoculated only with TBSV genomic RNA developed lethal systemic necrosis. Replication of the DIs was analyzed as in Figure 2. Upper panel shows an ethidium-stained gel, with the corresponding northern blot below. Numbered lanes refer to specific TBSV B10-DI clones. A typical PCR product and the T7 transcripts derived from it are indicated.

contained no promoter sequence and were also missing 2-4 nucleotides compared with the parental TBSV sequence. At the 3' end inserts lacked as few as none, but as many as 11 nucleotides. Polycytidine tails added prior to cloning also were an impediment. These problems were solved simultaneously by using the polymerase chain reaction (PCR) to correct and amplify cDNAs used for transcription. As outlined in Figure 4, the PCR strategy used one oligonucleotide primer containing the sequence for a bacteriophage T7 promoter fused to the 5' terminal 24 nt of TBSV and another primer specifying the complement to the viral 3' terminal 28 nt. Conditions for PCR were identical to those used to modify and clone the termini of TBSV and have been described elsewhere (Hearne, *et al.*, 1990). Transcripts of B10 DI cDNAs were made from 1 µg of PCR-amplified product in 100 µl reactions essentially according to the method of Janda, *et al.* (1987), but omitting the GpppG 5' cap analogue. Transcripts replicated in plants co-inoculated with TBSV RNA or virions. DI RNAs accumulated to high titers in the presence of helper virus, but no accumulation of DI was observed when transcripts were inoculated without TBSV (Figure 3). No differences in symptom attenuation were observed in plants when transcripts from DI clones or the uncloned DI RNA was co-inoculated with helper virus.

DI EVOLUTION

During the serial passage experiment it was noted previously that DIs appeared at different times in each of the individual high m.o.i. lines and that by passage 10 each had a distinctive size. We selected 2 lines (B10 and C6) in which the DIs appeared early to evaluate more thoroughly each step in the passage experiment. Interestingly, the predominant DI species present in each population after ten weeks of passage were noticeably smaller than that observed when the DI first appeared, suggesting evolution had occurred. Figure 4 (bottom panel) shows the DI RNA species present in the LiCl soluble fraction of RNA extracted from B10 infected plants at passages 3 (no DI), 4 and 10. The B10 DI first appeared during the 4th passage in *N. benthamiana* and consisted of a largely heterogeneous group of RNA species from approximately 300-800 b, with a predominant band at ~780b. After 10 passes, there was less heterogeneity, and the predominant DI species was approximately 600b. Cloning and sequencing of the DI populations at each passage was accomplished directly from the LiCl fractions by initial cDNA synthesis followed by PCR amplification as outlined in Figure 4. Primers used for cloning were similar to those used for preparing transcription templates except that unique restriction sites were incorporated into each terminus to facilitate cloning. Clones were obtained from PCR amplification of cDNA from the equivalent of as little as 8 µg of infected tissue.

A summary of the results of the sequence analysis is presented in Figure 5 for both the original and evolved C6 and B10 DI populations. These results represent the consensus of 18 DI clones from 4 separate populations. It is notable that each comprised essentially the same regions of sequence with some minor variations. Only two clones (from C6, pass 3) were identical in sequence suggesting that they might have resulted from PCR amplification of the same template. Minor differences in each of the other sequences suggest each may have originated separately, rather than by duplication of an original cDNA template. A major difference between C6 and B10 was the location of the genomic deletion defining regions I and II. All of the C6 DIs had a precise deletion between nt 178 to 1285, whereas the comparable deletion for all B10 DIs was between nt 168 to 1285. The C6 population was also distinctive in that it contained a greater portion of the polymerase domain (region II) than B10. A comparison of all of the DIs sequenced shows that the 5'-proximal junction of region II is generated with precision while the 3'-proximal portion tolerates more extensive sequence heterogeneity. Deletions resulting in the creation of regions III and IV in the DI RNAs also appeared to occur with precision as the junctions in all but the least-evolved (B10, pass 4) and most evolved (DI1) sequences were highly conserved. In general, the sequence differences between pass 3 or 4 and pass 10 involve heterogeneity at the 3'-end of region II and the precise deletion defining the 5' terminus of region III. These observations strongly support the idea that the DIs continued to evolve by additional deletion events from previously existing DIs.

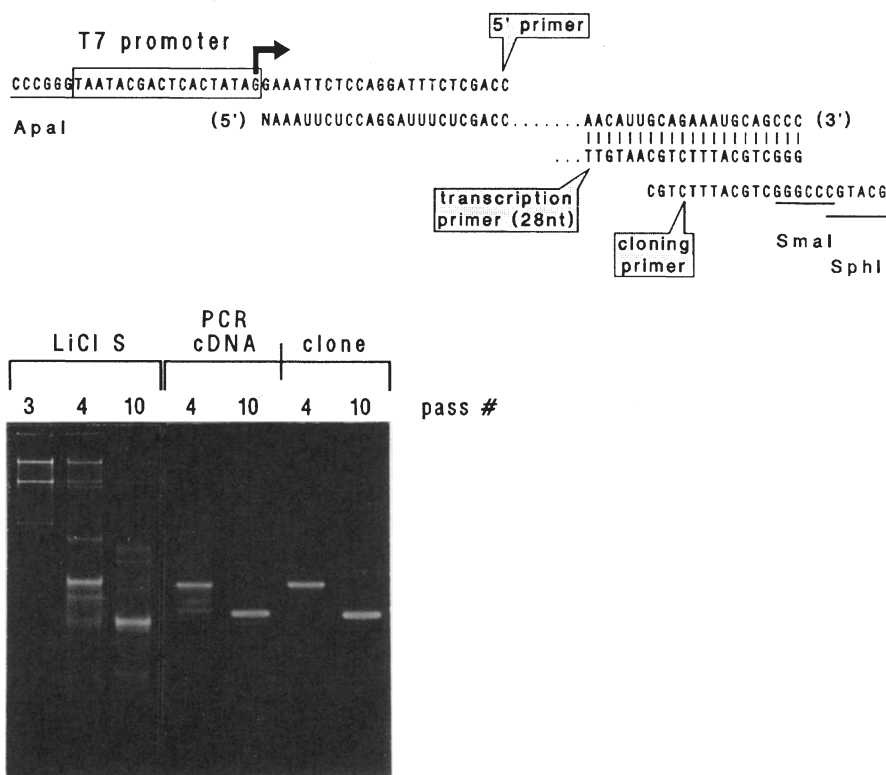


Figure 4. PCR strategy for cloning TBSV B10 DI RNAs. Upper panel shows sequences of primers used for cDNA synthesis and PCR amplification. The termini of TBSV template sequences are labelled (5' and 3'). Restriction sites, T7 promoter region, and transcription start site (solid arrow) are indicated. In the lower panel is an ethidium bromide-stained gel showing LiCl supernatant RNAs (LiS) isolated from passes 3, 4, and 10 (left 3 lanes). The center two lanes show PCR-amplified cDNAs resulting from passes 4 and 10. In the right two lanes are examples of inserts from clones generated from the amplified cDNAs. The identical strategy was employed to make clones from the TBSV C6 isolate after 3 and 10 passes.

DISCUSSION

These experiments confirm the *de novo* generation of DIs during serial passage of TBSV. It appears that two relatively precise deletion events take place giving rise to DIs with a highly conserved sequence motif in which most of the sequence heterogeneity within a given population is confined to the 3'-end of region II. The slight variations in the sequences between and within independently-derived DI populations indicate they may arise from separate events. There are at least two possible explanations for the strong conservation of sequences observed in TBSV DIs. One is that the mechanism of recombination is rigidly controlled, resulting in the generation of only certain deletions. Another explanation is that random deletions may occur during replication, but by selection, only templates with optimum abilities for replication are maintained. In actuality, a combination of these two explanations may operate. For instance, in the coronavirus mouse hepatitis virus (MHV), it is thought that deletions leading to formation of DIs involve recombination at conserved

elements of secondary structure (Makino, *et al.*, 1988). However, once generated, survival of the resulting recombinants depends upon maintaining the ability to be replicated. Although a similar mechanism may operate in creating TBSV DIs, searches for conserved elements of predicted secondary structure surrounding points of recombination resembling those of MHV, have so far been negative. Nevertheless, it seems clear that DIs contain elements required in *cis* for replication. That DIs maintain both viral termini, is not surprising since each is considered to be important in initiating complementary strand synthesis during replication. It is also not unprecedented for internal elements to be maintained. The intergenic region of brome mosaic virus RNA3 contains is important for efficient RNA replication (French and Ahlquist, 1987). In addition, it has been noted that animal virus DIs often contain internal regions of sequence (Makino, 1988; Schlesinger, 1988).

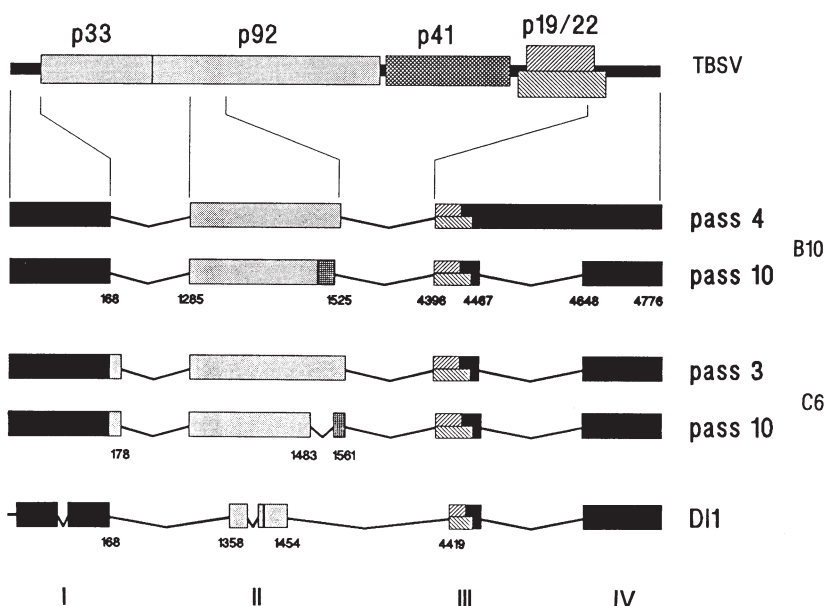


Figure 5. Evolution of *de novo* generated TBSV DI RNAs. A map of the TBSV genome is shown above with coding regions as shaded rectangles and the sizes of their protein products in kDa above; non-coding regions are black. Separate diagrams for the consensus sequence of each DI population in this study are given below with identical blocks of sequence in the same vertical position. Fine lines give the positions for each region of DI relative to the TBSV genome. Pass 3 and pass 4 represent sequences from initial populations of TBSV isolates C6 and B10 respectively, while pass 10 represents the passage at which the experiment was stopped. Precise definition of regions I-IV are given in the text. Numbers indicate consensus break points between different regions for some populations. A spontaneous DI characterized previously, (DI1) is provided for comparison.

The sequence motifs of TBSV DIs resemble in several ways those of the similarly-sized DIs recently characterized in association with TCV (Li *et al.*, 1989). In both viruses, DI populations have been generated *de novo* and have been shown to retain all of the 5' non-coding region and a precisely modified 3' non-coding region

consisting of the terminal 130-150 nt fused to variable portions of the C-terminus of the 3'-most ORF. An interesting distinction between the two types is the significant modification of the 5'-terminal region in the evolved TCV DIs and the absence of a significant region of internal sequence from the viral polymerase gene. These comparisons provide useful clues about sequences important in replication. To emphasize this, we note that the TCV DI RNAs have recently been useful for defining possible encapsidation signals through the use of RNase protection assays to identify regions of coat protein affinity (Wei *et al.*, 1990).

Plant virus DIs may prove useful in developing novel strategies for controlling plant virus diseases. In California, significant economic losses occur from tomato decline, a disease in which TBSV infection appears to play a major role (J. Gerik, unpublished results). In an effort to control this disease we are currently engineering transgenic tomato plants to express DI RNAs. This strategy is plausible because preliminary trials have demonstrated that TBSV DIs can function *in trans* to attenuate virus symptoms in tomatoes. This approach is similar to other methods which have been advanced for controlling virus diseases by engineering expression of virus-derived sequences into plants such as coat protein-mediated protection (reviewed in Register, *et al.*, 1989), expression of satellite RNAs (reviewed in Baulcombe, *et al.*, 1989), and anti-sense RNA expression (Loesch-Fries, 1987; Hemenway, 1988). Potential advantages of using DIs are that expression of foreign proteins is not required and the levels of constitutive expression necessary to achieve protection are expected to be low. We expect that many more plant viruses will be shown to produce DIs. In the future, based on a more thorough understanding of the mechanisms by which DIs are generated and maintained, it may be possible to design DI-like molecules to control viruses that may not normally make DIs.

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